Pollination and plant defence traits co-vary in Western Australian *Hakeas*

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Summary

• Despite the conflicting demands imposed by mutualistic (pollination) and antagonistic (florivory) processes, the relative importance of the key selective pressures influencing floral evolution are not readily apparent. In this study we quantified a range of floral and foliar traits within the genus *Hakea* to investigate how pollinator and herbivore selection might influence the evolution of floral attraction and defence attributes.

• Plant material was collected from populations of 51 Australian *Hakea* species native to southwestern Australia, and measurements were taken of foliage and inflorescence morphology, inflorescence colour and floral chemical defence. Hakeas were separated into bird- vs insect-pollinated species on the basis of stigma–nectary distance.

• Our results show how the evolution of insect vs bird pollination is closely linked to whether inflorescences are protected by physical (leaf spines, dense foliage) or chemical (floral cyanide) defences, respectively.

• Rather than being constrained by the necessity to attract pollinators, we suggest that pre-existing adaptations to combat florivore and herbivore attack directed the evolution of floral characteristics employed to attract pollinators and deter florivores. The inter-correlation among bird pollination, red flower colour and floral cyanide indicates floral coloration may signal to vertebrate florivores that the inflorescences are unpalatable despite their high accessibility.

Introduction

Conventionally assumed to have evolved solely for the purpose of pollinator attraction, it is increasingly apparent that floral traits evolve in response to a host of unrelated selection pressures (Armbruster, 2002; McCall & Irwin, 2006). Many flowers, for example, are imbued with chemical or physical deterrents that reduce access to would-be nectar robbers and florivores, deterrents that often impose a significant compromise on the development and expression of traits geared to pollinator attraction (McCall & Irwin, 2006; Ashman & Penet, 2007). Plant structures associated with reproduction are widely assumed to benefit from the possession of chemical and/or structural defences; indeed this is a fundamental tenet of the Optimal Defence Hypothesis (Rhoades, 1979). Nevertheless, in comparison with other aspects of plant–herbivore interactions, anti-florivore defence has received remarkably little attention, despite the obvious potential for floral herbivory to influence plant reproductive success and the evolution of floral defence (McCall & Irwin, 2006).

In terms of the amounts of material consumed, florivory can be as extensive as leaf herbivory (McCall & Irwin, 2006). Tobler *et al.* (2006) showed how over 80% of *Iris hexagona* inflorescences were consumed by white-tailed deer (*Odocoileus virginianus*) in the marshlands of the Mississippi delta, even though foliage was seldom grazed. The magnitude of such losses can impose direct costs to plants, particularly in resource-limited environments. Flowers represent a major resource sink and their damage may prevent reallocation of nitrogen or phosphorus following flowering, while at the same time increasing the rate of water loss through transpiration (Ashman, 1994; McCall & Irwin, 2006). However, it is the

potential for direct interference with reproduction that makes florivory such a potent selective force. Invertebrate florivory, while characterized by localized damage to floral tissues, has the capacity to lessen the attractiveness of flowers to pollinating insects by reducing petal area or pollen and nectar rewards (Leavitt & Robertson, 2006). Large vertebrates, on the other hand, may eliminate the entire reproductive potential of individual plants by removing flowers in their entirety, as Tobler *et al.* (2006) demonstrated when they recorded 20 times more mature seed capsules on *Iris hexagona* plants protected from white-tailed deer.

Sustained damage to flowers and the consequential limitations on plant reproductive performance might be expected to favour the development and expression of anti-florivore defence (McCall & Irwin, 2006; Hanley *et al.*, 2007). Indeed, because petals and sepals share a common lineage as modified leaves (Gutierrez-Cortines & Davies, 2000), there is a persuasive evolutionary reason why both should display the chemical and physical defences more normally associated with leaf tissues (McCall & Irwin, 2006). Plants whose flowers contain high concentrations of secondary compounds may exhibit enhanced resistance to florivory. For example, Adler *et al.*, (2001) showed how *Castilleja indivisa* inflorescences containing higher concentrations of alkaloids suffered less florivory than conspecifics with lower concentrations of alkaloids. Moreover, several studies have documented induced chemical resistance following damage to floral tissues (Strauss *et al.*, 2004; McCall, 2006). Although there has been negligible experimental examination of the subject (McCall & Irwin, 2006; Hanley *et al.*, 2007), structural defences may also play a part in florivore deterrence. Trichomes commonly occur inside flowers (Armbruster, 1997; Werker, 2000) and may limit invertebrate access to reproductive tissues. Leaf bracts or even spines may also protect flowers against florivory (McCall & Irwin, 2006; Hanley *et al.*, 2007).

One major constraint on the deployment of anti-florivore defences is that selection will act towards ensuring adequate pollinator access to flowers. Traits that deter florivores may also reduce pollinator visitation (Strauss *et al.*, 2002) and consequently plants may face a trade-off between the expression of anti-florivore traits and the expression of those that facilitate effective pollinator access (Ashman *et al.*, 2004). A further consideration in this relationship is the type of vector relied upon by the plant to carry pollen. Bird-pollinated inflorescences are often large, red-coloured and conspicuously located on the plant (Raven, 1972; Armstrong, 1979; Thompson & Wilson, 2008), characteristics that may attract and facilitate easy pollinator access but which also render flowers more apparent to potential florivores (also often birds). Similarly, wind-pollinated flowers must be able to easily release and receive pollen transported through the air, and inflorescences are thus often prominently positioned. By contrast, insect-pollinated species may be able to conceal their flowers from large vertebrate herbivores, while allowing access to the smaller invertebrates upon which cross-pollination depends.

Despite an increasing interest in the subject of florivory and floral defence, there is no clear picture as to whether the expression of chemical and physical deterrents is linked to the types of pollinator attracted to the inflorescence. The aim of our study was to determine whether the exposed inflorescences of bird-pollinated Western Australian *Hakea* (Proteaceae) species contain enhanced chemical defences when compared with insect-pollinated congeners, whose inflorescences are often protected by dense layers of spinescent foliage. Hakea species are characterized by their extremely spiny foliage. Nevertheless, there is considerable variation in the expression of spinescence within the genus, ranging from the highly spinescent, needle-leaved *Hakea psilorrhyncha* to broad-leaved, spineless species such as *H. laurina* (Fig. 1). Hakeas also exhibit marked variation in inflorescence morphology and type of pollinators attracted (Barker *et al.*, 1999), and in the deployment of chemical defences, especially cyanogens, against herbivores (Swenson *et al.*, 1989; Lamont, 1993).

Many *Hakea* species produce small axillary flowers held within a tight barrier of protective spines, apparently readily accessible only to insects (Fig. 1). These flowers also tend to be white, yellow or cream in colour (Barker *et al.*, 1999). Other species, by contrast, possess large inflorescences, usually red in colour, that are conspicuously located on the plant and readily accessible to avian pollinators. However omnivorous birds, such as black cockatoos (*Calyptorhynchus* spp.) and emus (*Dromaius novaehollandiae*), also have access to these exposed inflorescences (van Leeuwen & Lamont, 1996; Johnstone & Storr, 1998). Indeed, hakea flowers and fruits have been found in emu droppings (Davies, 2002; Calviño-Cancela *et al.*, 2006), while foliar and fruit traits in the genus have also been attributed to the evolutionary pressure imposed by cockatoos (Groom *et al.*, 1994; Groom & Lamont, 1997). Consequently, while the inflorescences of bird-pollinated hakeas may be more accessible than insect-pollinated species to allow pollinator access, they may at the same time be more accessible to avian florivores. The increased apparency (large, red inflorescences) and accessibility of bird-pollinated flowers may therefore render them more susceptible to florivore damage, unless they are simultaneously protected by chemical defences. In hakeas this defence is most likely in the form of cyanogenesis (Swenson *et al.*, 1989; Lamont, 1993).

In this study we characterized the leaf and floral morphologies of 51 *Hakea* species, divided them into insect- and bird-pollinated groups, quantified floral cyanogen concentrations and devised an accessibility index to quantify pollinator and florivore access to the inflorescences of each species. We then tested the following deductive (directional) hypotheses: (1) species with prominently displayed flowers (high accessibility) are bird-pollinated (Armstrong, 1979; Thompson & Wilson, 2008); (2) highly accessible flowers are cyanogenic (Lamont, 1993; Adler *et al.*, 2001); (3) highly accessible

Fig. 1 Six Western Australian *Hakea* species representing the wide range of leaf and floral morphologies present within the genus. Species a–c (*H. orthorrhycha*, *H. cucullata* and *H. laurina*, respectively) are putatively bird-pollinated, while d–f (*H. brownii*, *H. cygna* and *H. psilorrhyncha*, respectively) are putatively insect-pollinated.

flowers are red (Armstrong, 1979; Thompson & Wilson, 2008); (4) red flowers are cyanogenic (from hypotheses 2 and 3); (5) species with red flowers are bird-pollinated (Raven, 1972; Thompson & Wilson, 2008).

Materials and Methods

Study system and collection of plant material

Plant material was collected from southwestern Australia between October 2003 and June 2005. Six individual plants were selected from populations of 51 species, and from these, measurements of foliage and inflorescence morphology, inflorescence colour and samples for chemical (cyanide) analysis were taken (see Supporting Information, Table S1). Species were chosen to represent a wide range of these variables and cover as many of the infrageneric groupings (Barker *et al.*, 1999) as possible.

Classification of bird and insect pollination

Hakea species were assigned as either bird- or insect-pollinated, as no mammal-pollinated species are recognized and insufficient information was available to discriminate between different

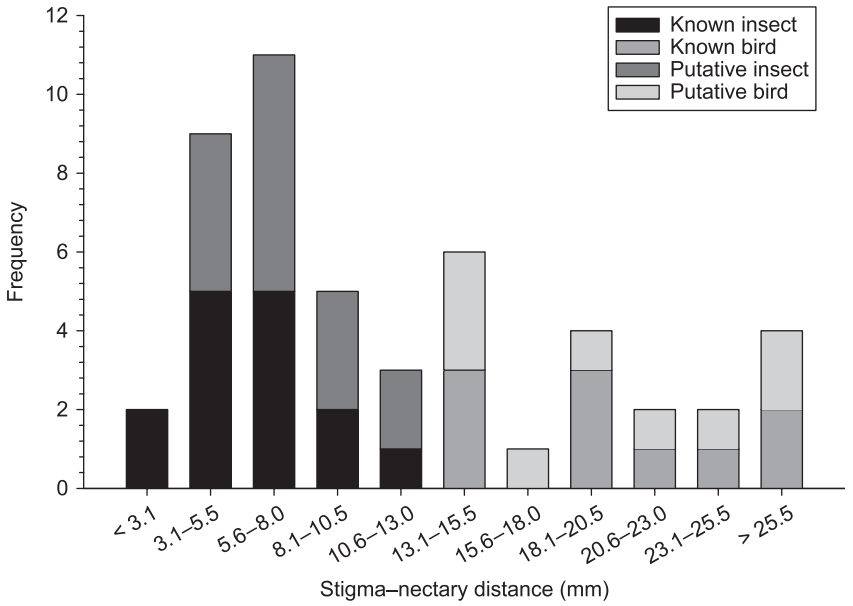

Fig. 2 Frequency of insect- and bird-pollinated Western Australian *Hakea* species classified by distance between the stigma and nectary. The frequency of putative insect- and bird-pollinated species, classified on the basis of stigma–nectary distance (insect < 13 mm > bird), is also shown.

invertebrate pollinators. For 25 species, pollinator type was assigned from the field observations of Brown *et al.* (1997), Lamont *et al.* (1987, 1998) and Houston (2000) (Table S1) before being related to mean stigma–nectary distances (SND – determined using Vernier callipers for one flower from each of six inflorescences on each plant sampled), using logistic regression. This strongly bimodal frequency relationship (Fig. 2) was tested in a phylogenetic context using BayesTraits: discrete (Pagel & Meade, 2007) and found to be highly significant (likelihood ratio range across 10 randomly selected maximally parsimonious trees = 26.98–27.46, $P < 0.001$). The circumflexion point thus obtained was used to classify the remaining species for which we lacked direct observations into likely bird-pollinated (> 13 mm SND) vs likely insect-pollinated (< 13 mm SND).

Corroboration for our use of SND to classify pollinator type was provided by the fact that shortest bill length among the principal avian pollinators in Australia (honeyeaters; Meliphagidae) is 12 mm (Paton & Ford, 1977). Since pollen is likely to be transported via the head feathers, the SND of bird-pollinated species can be expected to exceed this length. Moreover, the stigma is remote from the perianth in all hakeas (Fig. 1) so that insects shorter than a critical body length would not touch the stigma while feeding from the nectary. Insect pollinators rarely exceed a body length of 15 mm (B. Lamont, unpublished) and there are no long-tongued bees native to Western Australia (Houston, 2000). Thus it is unlikely that insect-pollinated species could feasibly have a SND in excess of 15 mm. Flower colour was assessed in the field using the Royal Horticultural Society (UK) colour chart (Table S1). Species designated as having 'red' flowers included those categorized as deep pink by the RHS scheme. All other colours were designated 'nonred'.

Floral chemical defence

Of the principal secondary metabolites linked with plant defence, only phenolic and cyanogenic compounds are known in the Proteaceae (Swenson *et al.*, 1989; Rafferty *et al.*, 2005), the latter most commonly associated with reproductive tissues (Lamont, 1993). Cyanogenic compounds are frequently linked to anti-herbivore plant defence, a role known to include protection against avian herbivores (Hansen *et al.* 2004). Field assessment of floral cyanide concentration was achieved by placing 0.1 g of freshly harvested material in a 10 ml screw-top vial. Three drops of deionized water were added and the material crushed with a metal rod. Feigl-Anger test paper, cut into a strip 60 mm long and 5 mm wide, was suspended just above the mixture in each vial via the cap, such that a 40 mm length of the paper was exposed in the vial. Samples were left for 2 h at ambient temperature, the presence of released hydrogen cyanide indicated by the test paper turning blue. Pencil marks at 5 mm intervals along the exposed 40 mm length of paper facilitated a semi-quantitative analysis of floral cyanide concentrations; scores of 0–10 were allocated for successive segments of the paper that turned blue following exposure to cyanide (Lamont, 1993). Feigl-Anger tests were replicated six times for each species (Table S1).

For 14 *Hakea* species sampled in 2003 we conducted more rigorous quantitative assessments of floral cyanide concentrations. Inflorescence material was collected from six individual plants in the field and transported and stored in refrigerated conditions for no more than 2 d after collection. Total cyanides were then determined by automated colorimetry following acid hydrolysis of a 1 g sample of ground inflorescence material and separation of the cyanide by distillation (Clesceri

et al., 1998). A correlation of floral cyanide concentrations using both laboratory and field methods revealed a close positive relationship ($r^2 = 0.69$, $F_{1,13} = 28.21$, $P < 0.001$). Thus we concluded that the field method was robust enough to discriminate between species with 'low' (FA value of 5 or less, ≤ 1 μg g^{-1} cyanide) and 'high' (FA value of 7.5 or more, ≥ 10 μg g^{-1} cyanide) floral cyanide concentrations. To determine whether phenolics were present in hakea inflorescences, 1 g of ground inflorescence material was analysed following the Folin–Denis method (Waterman & Mole, 1994). Phenolics were present only in low quantities and showed no relationship with cyanide concentration ($r^2 = 0.16$, $F_{1,12} = 2.39$, $P = 0.148$). Thus we concluded that phenolics played little part in floral defence and undertook no further analyses.

Floral accessibility

In order to quantify how structural defences influence the accessibility of inflorescences to herbivores, we devised an index based on measurements of inflorescence length and width, the length of stem-bearing inflorescences, leaf area, the number of spines present on each leaf, spine sharpness (length × basal width) and the number of leaves present on the length of stem-bearing inflorescences. The index was based on the following logic and evidence: larger inflorescences are more visible and accessible to pollinators and florivores (van Leeuwen & Lamont, 1996; Barker *et al.*, 1999); sparser leaves (fewer leaves per unit length of flowering stem) surrounding the inflorescences (always produced in the leaf axils) increase floral visibility and accessibility; and longer internodal distances and larger leaves provide perches and landing platforms for birds (Barker *et al.*, 1999) (there are no hovering birds in Australia). Fewer and blunter spines, by contrast, also increase accessibility to vertebrates (Hanley *et al.*, 2007). Inflorescence length and width were quantified using Vernier callipers for a minimum of three inflorescences from each of six plants per species. The area of individual leaves was determined using an iMac, Epson 11260 USR scanner and imageJ software for one leaf sampled from each of six individual plants per species. The number of spines present on each leaf was also noted. Spine length divided by basal width (to give spine sharpness) was calculated from Vernier calliper measurements of all spines present on a minimum of four leaves per plant per species. The accessibility index (AI_{log10}) was calculated as follows: (inflorescence length × width × length of flower-bearing stem × leaf area)/(leaf number on flower-bearing stem × spine number × spine sharpness). The accessibility index for needle-leaved species was divided by two to account for their extra stiffness since their leaf mass area is about twice that of broad leaves (Groom *et al.*, 1997). Species were categorized as having 'high' ($AI_{log10} \geq 5.00$) or 'low' ($AI_{log10} \leq 4.99$) accessibility.

Relationships among pollinator type, floral accessibility and defence

All traits were also treated as qualitative with two classes (pollinator = 'insect' vs 'bird'; accessibility = 'high' vs 'low'; cyanide = 'high' vs 'low'; colour = 'red' vs 'nonred'). Correlated evolution of traits was tested with a Markov-chain model of trait-transition probabilities using estimated phylogeny and maximum likelihood ('omnibus' test of BayesTraits: discrete (Pagel, 1994; Pagel & Meade, 2007)). The analysis required a fully resolved tree and branch lengths, neither of which was available in the absence of a fully resolved, molecular phylogeny. However, using the phylogeny of Barker *et al.* (1999), based on 59 morphological characters for 88 of the 149 Australian *Hakea* species, we set branch lengths as equal and arbitrarily resolved the phylogeny using a stratified-haphazard method, generating 10 trees that represented the most divergent possible topologies (Fig. 3). Analyses considered all 10 topologies, and we recorded the range of outcomes. Further tests of ordered evolutionary change and contingent evolutionary changes were made by specifying equalities of transition frequencies in BayesTraits (Pagel, 1994; Armbruster, 2002).

Results

Bird-pollinated species (i.e. those categorized as having high SND) tended to exhibit a greater incidence of red flower colour (56% bird vs 13% insect) and 'high' accessibility (AI) (94% bird vs 35% insect) than insect-pollinated hakeas. At the same time, floral cyanide was more frequently categorized as 'high' in bird-pollinated (67%) species than in hakeas pollinated by insects (29%). When AI was compared with bird vs insect pollination (hypothesis 1) in a phylogenetic context (omnibus tests), a clear relationship was apparent: highly accessible inflorescences were much more commonly associated ($\chi^2 = 18.4$–19.0; $P < 0.001$ for all 10 topologies) with bird-pollinated species (Table 1). We also examined the relationship between floral chemical defence (cyanide concentration) and AI (hypothesis 2). Although only significant at the $P = 0.06$–0.09 level, there was some support for a positive association between inflorescence accessibility and high floral cyanide concentration. Omnibus tests also revealed a consistent association between high floral cyanogenic concentrations and bird pollination ($\chi^2 = 10.4$–11.4; $P < 0.05$), providing further support for the putative interaction between high floral accessibility and cyanide concentration. Further tests of ordered evolutionary change and contingent evolutionary change (Pagel, 1994; Armbruster, 2002) indicated that the evolution of bird pollination occurred primarily in insect-pollinated lineages with an existing capacity for floral cyanide production; that is, bird pollination evolved only in cyanogenic, insect-pollinated lineages (Table 1).

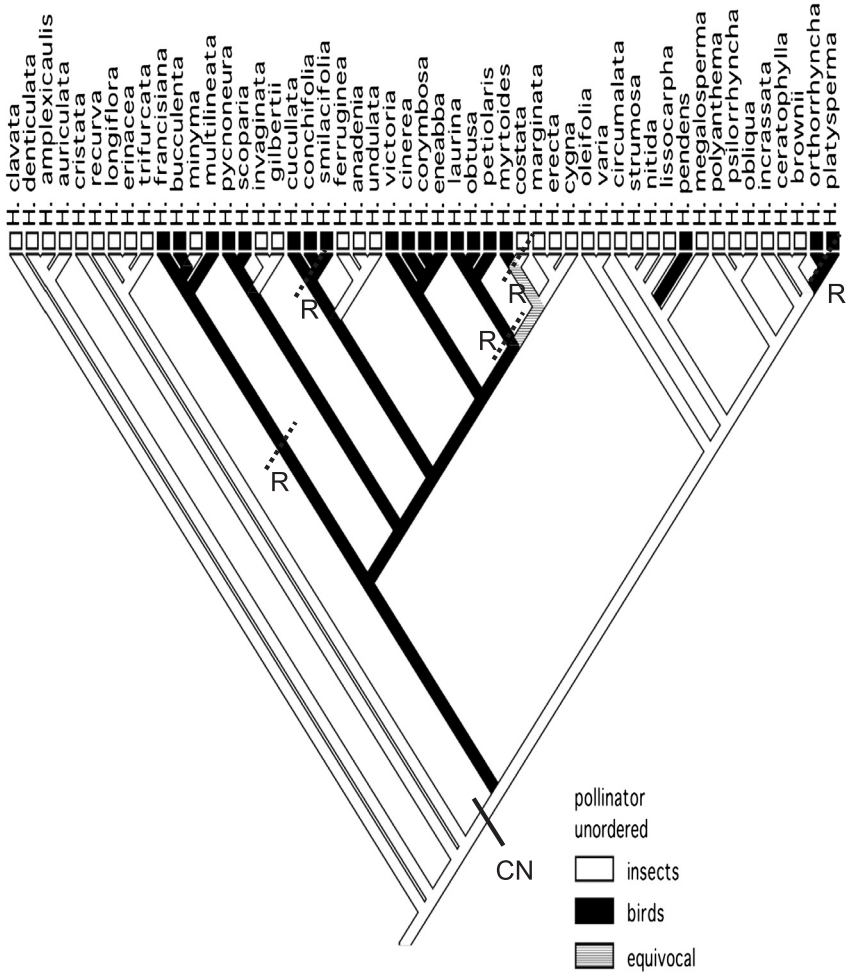

Fig. 3 Multiple origins of bird pollination (black shading) and red flowers (R, dotted diagonal slashes) traced on to a representative, resolved, maximally parsimonious tree for 51 *Hakea* species. The solid diagonal slash indicates a single prior origin of cyanide production (CN). For simplicity, losses of red flowers and cyanide production are not shown. Phylogeny based on Barker *et al*. (1999).

Omnibus tests revealed a positive association between red flowers and high floral cyanide concentration ($\chi^2 = 9.3$–10.2; $P = 0.04$–0.06). Red inflorescences were also strongly associated with high floral accessibility ($\chi^2 = 13.9$–14.1; $P < 0.01$), and pollination by birds ($\chi^2 = 19.7$–21.3; $P < 0.001$). Thus we obtained support for hypotheses 3–5. A trait contingency test further suggested that nonred inflorescences evolved more readily in insect-pollinated *Hakea* species. The close association between flower colour and pollinator type we obtained provides strong support for our assigning pollinator categories based on SND, as red flowers are frequently associated with bird pollination (Raven, 1972; Rodriguez-Girones & Santamaria, 2004; Cronk & Ojeda, 2008). Similarly, when we explored the relationship between inflorescence length and SND using Pearson's correlation, we also found a positive relationship ($r^2 = 0.36$, $F_{1,48} = 26.20$, $P < 0.001$), supporting the commonly held assumption that larger inflorescences are more attractive to birds than insects (Cronk & Ojeda, 2008; Thompson & Wilson, 2008).

Species representing the infrageneric groupings of Barker *et al.* (1999) were used to compare floral trait characteristics in insect- and bird-pollinated hakeas, that is, within and between closely related species that diversified after the evolution of bird pollination (Table 2). Analyses showed that the inflorescences of bird-pollinated species were significantly larger, more cyanogenic, more accessible and had greater SND than insect-pollinated species. Thus we conclude that for these closely related species, there is consistent variation in floral and foliar traits relative to pollination syndrome, as well as providing further support for the hypothesis that highly accessible, bird-pollinated flowers contain enhanced chemical defences to deter potential florivores.

Discussion

There has been an increasing realization that floral evolution can only be properly understood when factors not directly associated with pollinator attraction are also considered as selective agents. In particular, relationships between mutualistic (pollinator attraction) and antagonistic (florivore deterrence) processes are expected to be pivotal in shaping floral trait evolution (Armbruster, 2002; McCall & Irwin, 2006;

Table 1 Summary of results for correlated trait evolution in Western Australian *Hakea* species

Trait comparison	Omnibus log-likelihood ratio (χ^2)	Evidence for correlated evolution?	Evidence of ordered evolution	Trait contingency 1	Trait contingency 2
Accessibility × pollinator	18.4–19.0	Yes ($P < 0.001$): species with very accessible flowers are bird-pollinated			
Accessibility × floral cyanide	8.1–8.5	Marginal ($P = 0.06$–0.09): accessible flowers tend to be cyanogenic			
Floral cyanide × pollinator	10.4–11.4	Yes ($P < 0.05$): species with cyanogenic flowers are bird-pollinated	Yes ($\chi^2 = 4.81$–1.70; $P = 0.03$–0.20): cyanogenesis evolves before shift to bird pollination	Yes ($\chi^2 = 4.86$–2.38; $P = 0.03$–0.12): cyanogenic species evolve bird pollination more frequently than acyanogenic	Marginal ($\chi^2 = 3.32$–2.62; $P = 0.07$–0.10): insect-pollinated lineages more likely to lose cyanide than bird-pollinated lineages
Flower colour × accessibility	13.9–14.1	Yes ($P < 0.01$): accessible flowers are red			
Flower colour × floral cyanide	9.3– 10.2	Yes ($P = 0.04$–0.06): red flowers tend to be cyanogenic			
Flower colour × pollinator	19.7–21.3	Yes ($P < 0.001$): species with red flowers are bird-pollinated			Yes ($\chi^2 = 4.94$–3.32; $P = 0.03$–0.07): insect-pollinated species evolve nonred flowers more readily than bird-pollinated species

Pollinator type, flower cyanide content, accessibility and colour were examined using omnibus, ordered evolution, and trait contingency tests (Pagel, 1994; Armbruster, 2002). The omnibus test is the maximum-likelihood ratio of two models differing by four terms (one-tailed χ^2, with 4 df). Ordered evolution and contingency tests are based on maximum-likelihood ratios of two models with one-term differences (one-tailed χ^2 with 1 df).

Ashman & Penet, 2007). Our analysis shows not only that highly accessible bird-pollinated species are better defended by floral cyanogens (hypothesis 1), but also that bird pollination could only have evolved from insect-pollinated taxa with a pre-existing capacity to protect inflorescences through the synthesis of floral cyanide. Our data also point to a link between floral accessibility (AI) and floral cyanogen concentrations (hypothesis 2). Indeed, had we taken Westoby & Wright's (2006) position that 'individual species represent independently competent units of replication in the search for trait combinations' and performed a nonphylogenetically corrected analysis, a strong association between high floral cyanide and high AI would be apparent. The expectation that highly accessible, bird-pollinated inflorescences should have well-developed chemical defences as an alternative to physical protection (low AI) against avian florivores to which they are equally accessible is further supported by the significant overall association between bird pollination and high floral cyanide content, and by the fact that we found consistent differences in floral accessibility and cyanide content within closely related infrageneric groups.

We also show how inflorescence colour is strongly associated with insect vs bird pollination, floral accessibility and defence (hypotheses 3–5). The close association between bird pollination and red flower colour is traditionally thought to have evolved through pollinator selection. Not only are birds highly responsive to red, but the colour is assumed to be inconspicuous to bees, reducing the possibility of their depleting nectar rewards without effecting pollen transfer between plants (Raven, 1972). However, this assumption is too simplistic: bees are known to detect and visit red flowers (Chittka & Waser, 1997; Hanley *et al.*, 2008). A recent, niche-based competition theory suggests that red flowers evolved as a signal to bees that nectar rewards are likely to be exploited, and depleted, by more efficient bird pollinators (Rodriguez-Girones & Santamaria, 2004). However, this hypothesis remains focused exclusively on pollinator selection. Observations of nectar-feeding insects on bird-pollinated Australian Proteaceae (Lamont & Collins, 1988) suggest that this reasoning may not hold for all bird-pollinated plant species.

We offer a different, although not mutually exclusive, explanation. On the one hand, red is a clear visual signal to nectivorous birds that an inflorescence is worth visiting (Lamont & Collins, 1988; Rodriguez-Girones & Santamaria, 2004; Thompson & Wilson, 2008), while on the other, red is a universal warning to avian predators, often causing them actively to reject potential prey items (Thomas *et al.*, 2004). Indeed, the red coloration of juvenile leaves in heterophyllous trees of the Mascarene Islands is thought to be an evolutionary response to selective herbivory by avian herbivores (Hansen *et al.*, 2004). Therefore, just as nectar-feeding birds can learn to associate red flowers with abundant nectar (Lamont & Collins, 1988), so avian florivores can learn to associate red with

Table 2 Comparison of stigma to nectary distance (SND), inflorescence size and accessibility (AI), and floral cyanide content, of insect-pollinated (I) and bird-pollinated (B) Western Australian *Hakea* species

		Floral character			
Species group	Species	SND (log_{10} mm)	Inflorescence size (log_{10} mm^3)	Accessibility (log_{10}AI)	Cyanide (arcsine)
1: Multilineata	*minyma* (I)	**0.775**	**3.915**	**6.21**	**18.43**
	bucculenta (B)	**1.289**	**5.347**	**6.81**	**18.43**
2: Sulcata	*gilbertii* (I)	0.959	3.764	3.32	0.0
	invaginata (I)	1.064	4.050	4.93	18.43
	Mean	**1.012**	**3.907**	**4.1**	**9.22**
	pycnoneura (B)	1.155	4.739	6.99	18.15
	scoparia (B)	1.179	4.775	6.82	18.43
	Mean	**1.167**	**4.757**	**6.9**	**18.29**
3: Undulata vs Cucullata	*anadenia* (I)	0.699	3.223	4.33	4.70
	ferruginea (I)	1.093	3.779	5.80	10.78
	undulata (I)	0.544	3.148	4.26	2.34
	Mean	**0.779**	**3.383**	**4.8**	**5.94**
	conchifolia (B)	1.423	4.687	5.17	18.43
	cucullata (B)	1.377	4.640	7.11	0.0
	smilacifolia (B)	1.155	3.252	5.08	6.80
	Mean	**1.318**	**4.193**	**5.8**	**8.41**
4: Ulicina	*costata* (I)	0.808	3.043	2.19	6.20
	myrtoides (B)	1.250	4.687	5.94	18.43
5: Ceratophylla vs Platysperma	*brownii* (I)	0.846	3.526	4.82	12.48
	ceratophylla (I)	0.851	3.607	5.18	7.77
	Mean	**0.849**	**3.567**	**5.0**	**10.13**
	orthorrhyncha (B)	1.403	4.649	7.13	18.43
	platysperma (B)	1.158	4.363	5.39	18.43
	Mean	**1.281**	**4.506**	**6.3**	**18.43**
Results of paired *t*-test	t_4	6.081	6.708	3.174	2.853
	P	0.004	0.003	0.034	0.046

Comparisons (one-tailed, paired *t*-tests) between closely related species were conducted using the infrageneric groups of Barker *et al.* (1999). Figures in bold denote values used in the paired *t*-tests.

the bitterness and toxicity of cyanogenic defence (Gleadow & Woodrow, 2002; Hansen *et al.*, 2004). This hypothesis is supported by: (i) our finding of a consistent positive association between high floral cyanide concentrations and bird pollination; and (ii) the comparative data suggesting that capacity to produce floral cyanogens evolved simultaneously with red coloration. The fact that cyanogenic compounds in hakeas are synthesized from tyrosine, rather than via the acetyl-CoA pathway from which the floral pigment anthocyanin is produced (Swenson *et al.*, 1989), also suggests that they have evolved independently. Since all red-flowered species are high in floral cyanide while other bird-pollinated species of different colours vary in cyanide concentration, there is some support for the evolution of red pigments as a cue to the presence of floral cyanide.

The relationships among inflorescence colour, accessibility, chemical defence and pollinator type seen in Western Australian *Hakea* provide strong support for the contention that floral evolution can only be understood when adaptive responses to nonpollinator agents of selection are considered in tandem with pollinators (McCall & Irwin, 2006; Ashman & Penet, 2007; Rausher, 2008). However, our study also suggests that pre-existing adaptations to combat foliar herbivory are important in constraining floral trait evolution. Because bird pollination evolved from a group of insect-pollinated plants with an existing capacity to defend their flowers through cyanogenesis, rather than through protection by physical defence (dense, spiny foliage), the evolution of bird pollination in the genus *Hakea* was constrained by variation in the spinescent morphology of ancestral species, that is, a trait that evolved as a response to foliar herbivory (Hanley *et al.*, 2007).

Molecular dating of avian clades shows that nectar-feeding birds (Meliphagidae) radiated in the early Eocene (Barker *et al.*, 2004). Although coincidental with the diversification of the Proteaceae (Barker *et al.*, 2007), the arrival of the Meliphagidae in Australia was pre-dated by the omnivorous bird families Casuariidae (cassowary and emu) and Cacatuidae (cockatoo) (Barker *et al.*, 2004), and by marsupials (Nilson *et al.*, 2004). These groups are important foliar and floral

herbivores whose feeding activity is reduced by the presence of structural defences such as spines in the Australian Proteaceae (Groom & Lamont, 1997; Hanley *et al.*, 2007). It is probable, therefore, that the Proteaceae had evolved highly spinescent foliage and chemically defended inflorescences well before specialized avian pollinators exerted a significant influence over floral trait evolution in the Australian Proteaceae. Having arrived in Australia, nectar-feeding Meliphagidae are likely to have exploited more accessible, insect-pollinated species. An interaction over 50 million yr since the Eocene has facilitated the evolution of greater accessibility (larger flowers and inflorescences, less spiny foliage, greater internode distance to allow perching) and increased floral chemical defence to compensate for reduced structural defences.

Although pollination syndromes provide a useful means of understanding the mechanisms of floral diversification, as Fenster *et al.* (2004) point out, there is a less than perfect correspondence between floral traits and the pollination syndrome that is supposed to have shaped them. In attempting to explain apparent divergence between present-day floral traits and pollinator syndrome, Fenster *et al.* (2004) invoked the concept of historicity; in essence, floral evolution follows 'lines of least resistance'. Thus, rather than following the paradigm that floral traits evolved as a consequence of pollinator selection, or the more recent suggestion that pre-adaptations to floral herbivory are important, for our Western Australian *Hakea* species the evolution and radiation of bird pollination seem also to have been shaped by pre-adaptations to foliar herbivory. Consequently, adaptations against both foliar and floral herbivory should be considered in the search for a clearer understanding of floral trait evolution.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Foliar and floral traits of Western Australian *Hakea* species

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